Nano-fluidic Proteomic Assay for Serial Analysis of Oncoprotein Activation in Clinical Specimens

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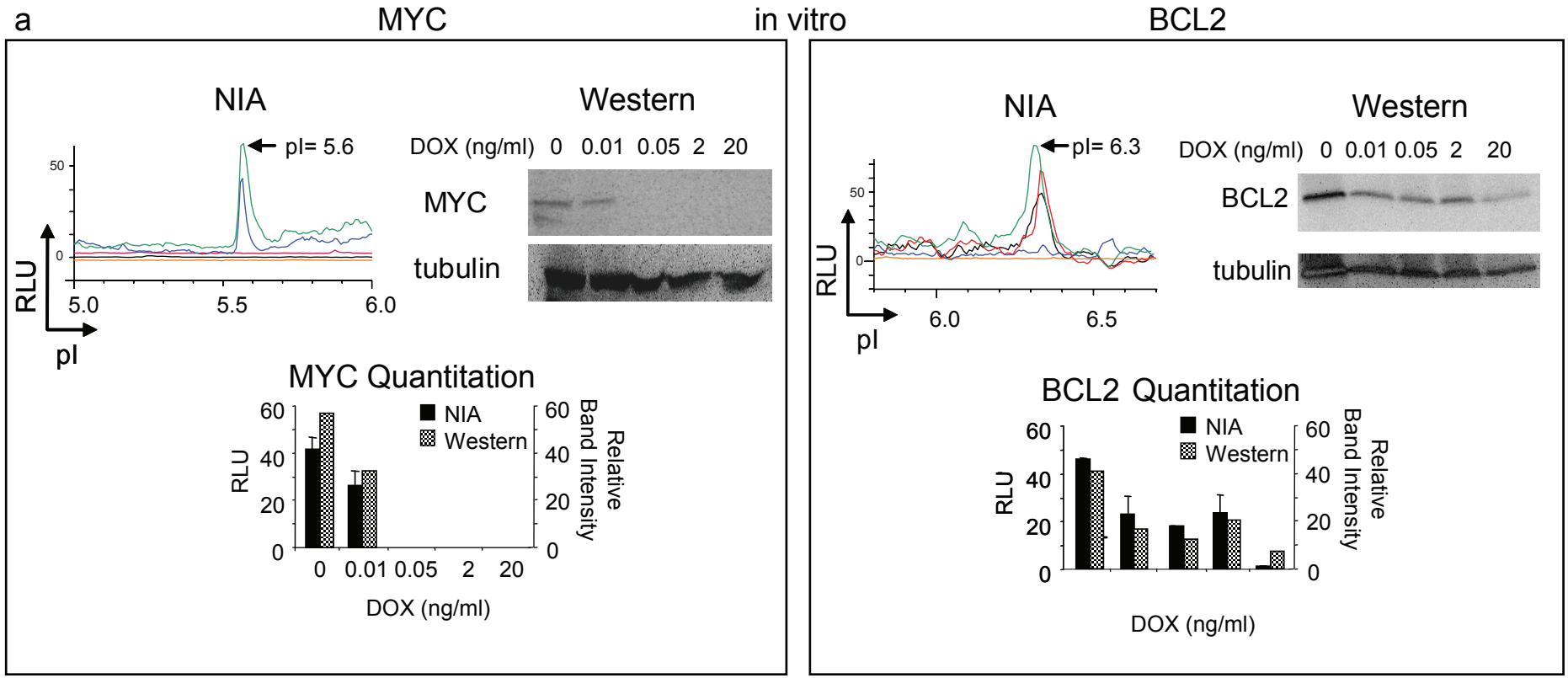
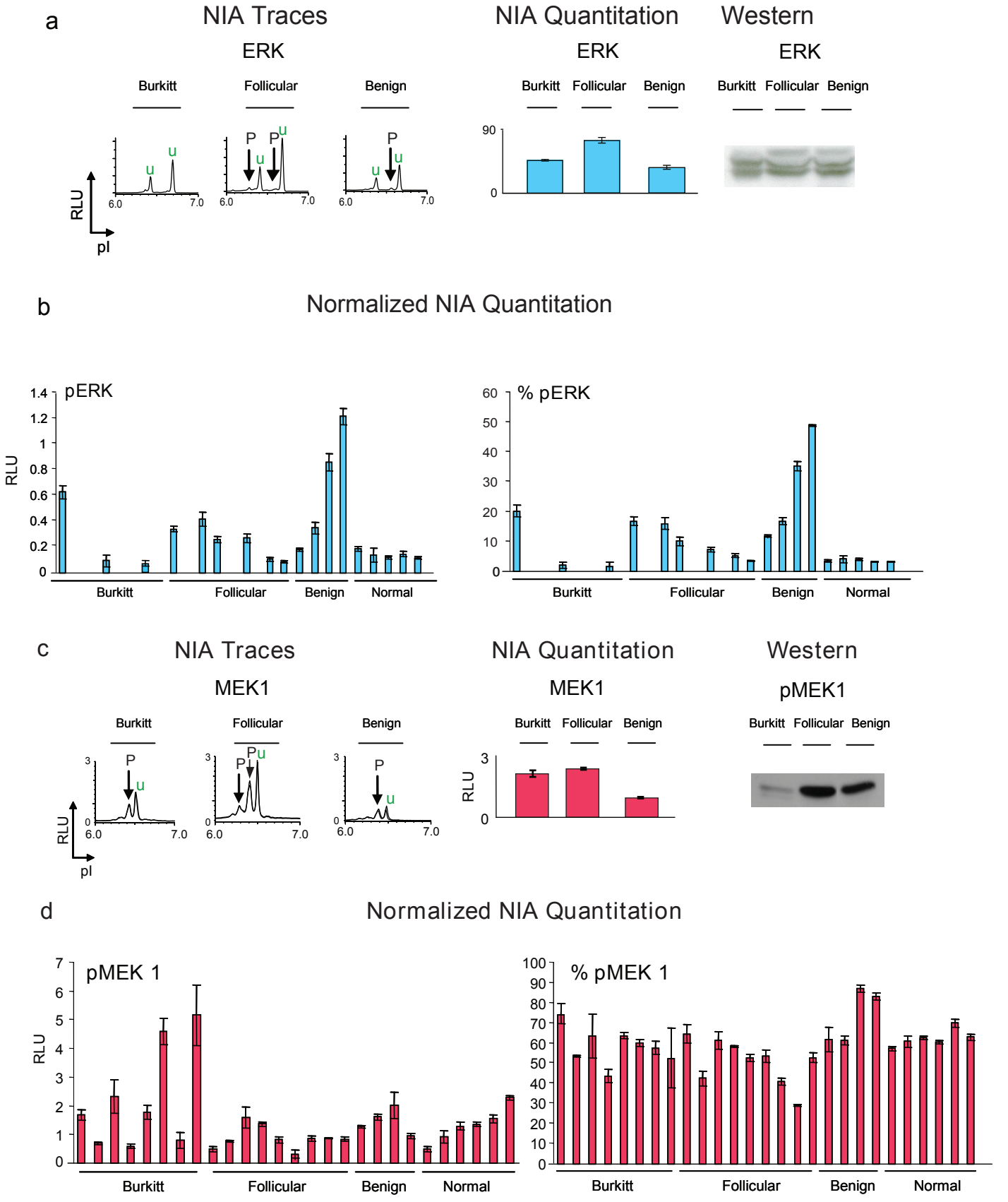
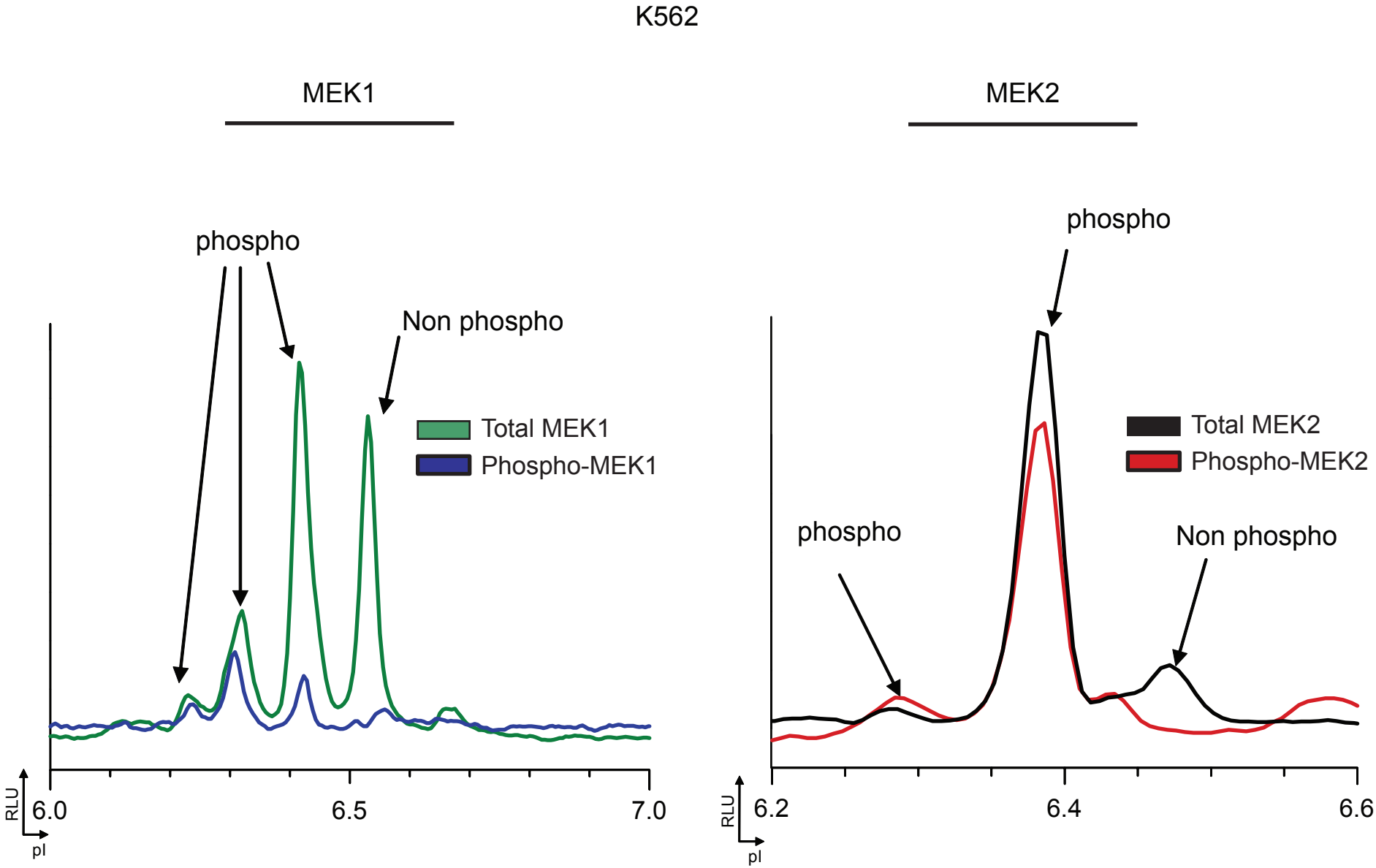


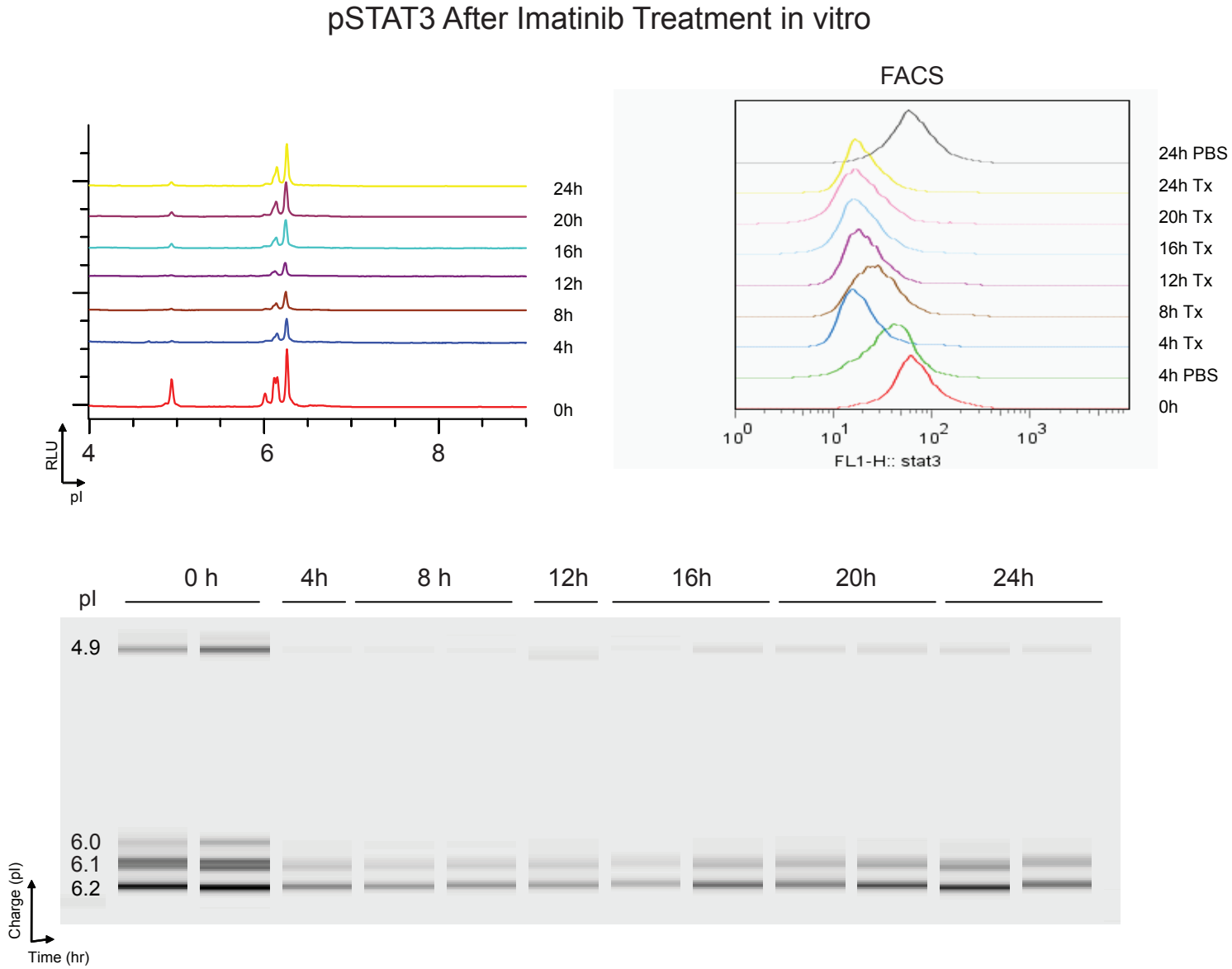
Supplementary figure 1. Sensitivity of NIA versus Western Blot for MYC and BCL2 in Cellular Lysates. a. The measurement of oncoprotein expression in transgenic mouse tumors. The Tet-off system was used to generate conditional transgenic mouse models of MYC or BCL2 induced lymphomagenesis. Using tumor derived cell lines from these transgenic models, we were able to conditionally regulate the levels of either MYC or BCL2 oncoprotein expression by titrating the concentration of doxycycline (dox) in their growth media. Representative NIA tracings are shown (green = dox 0 ng/ml, blue = 0.01 ng/ml, red = 0.05 ng/ml, black = 2 ng/ml, orange = 20 ng/ml). NIA peak height and western blot densitometer quantification are graphed. Corresponding analysis by Western blots is shown. Results obtained were statistically significant for MYC (Pearson correlation R= 0.99) and BCL2 (Pearson correlation R= 0.94).



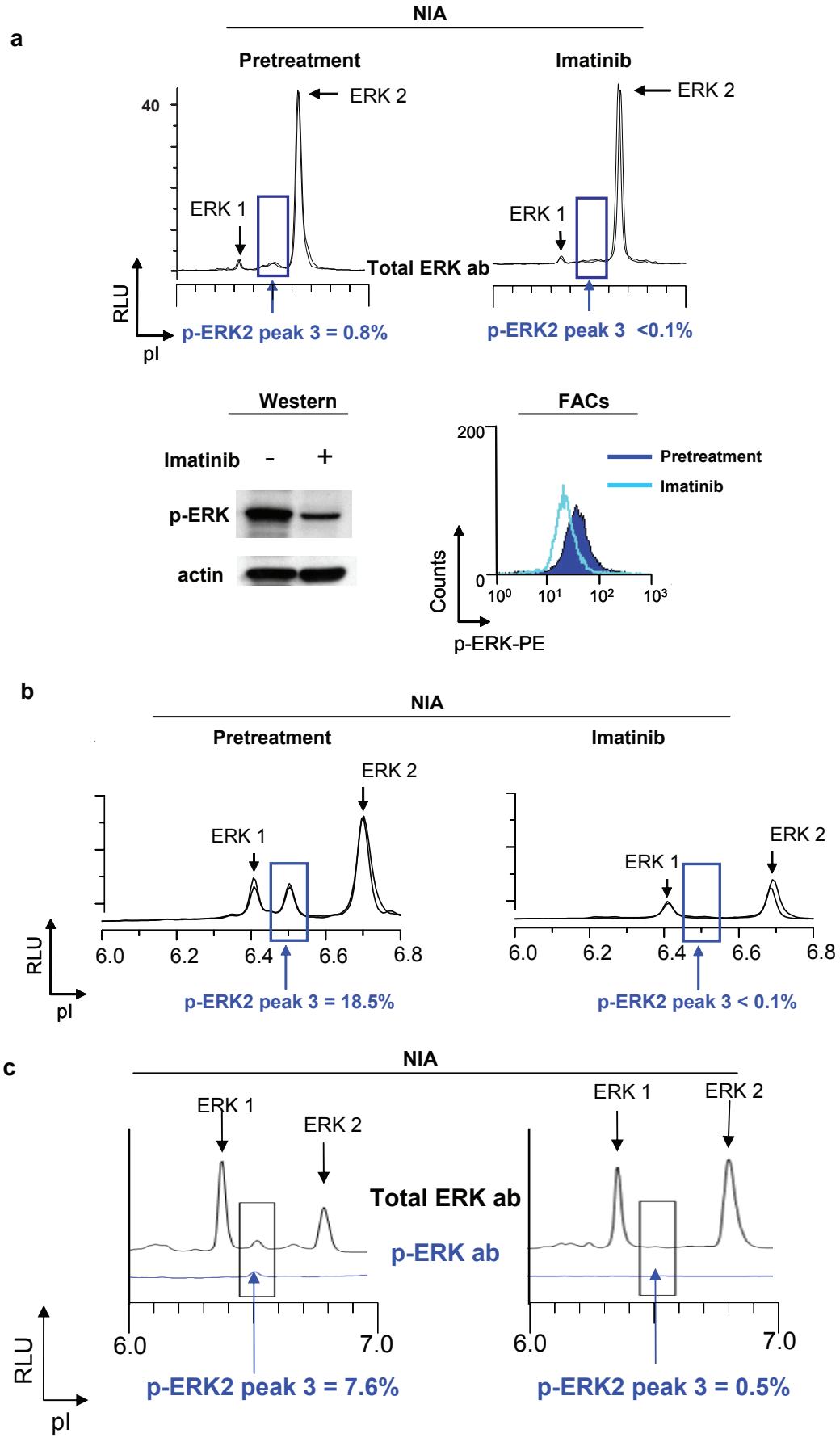
Supplementary figure 2. NIA Analysis of ERK and MEK1 Isoforms in a Panel of 27 Lymphoma and Control Specimens. NIA was used to measure phosphorylated and unphosphorylated isoforms of ERK and MEK1 in clinical patient specimens. a,c. Left: Representative NIA traces of total ERK and total MEK1 (phosphorylated isoforms are more cationic, black arrows. "p". Unphosphorylated isoforms are labeled "u" in green). Center: NIA quantification for total ERK and total MEK1. Right: Western blot for total ERK and phosphorylated MEK1 in representative Burkitt's lymphoma, follicular lymphoma, and benign lymph tissues are shown. b, d. Normalized NIA quantitation. NIA pERK and pMEK1 values were normalized against HSP70 values for each of 27 samples (left). Percent pERK and pMEK1 were calculated and graphed (right). All data is represented as the mean of four replicates per sample +/- standard error.



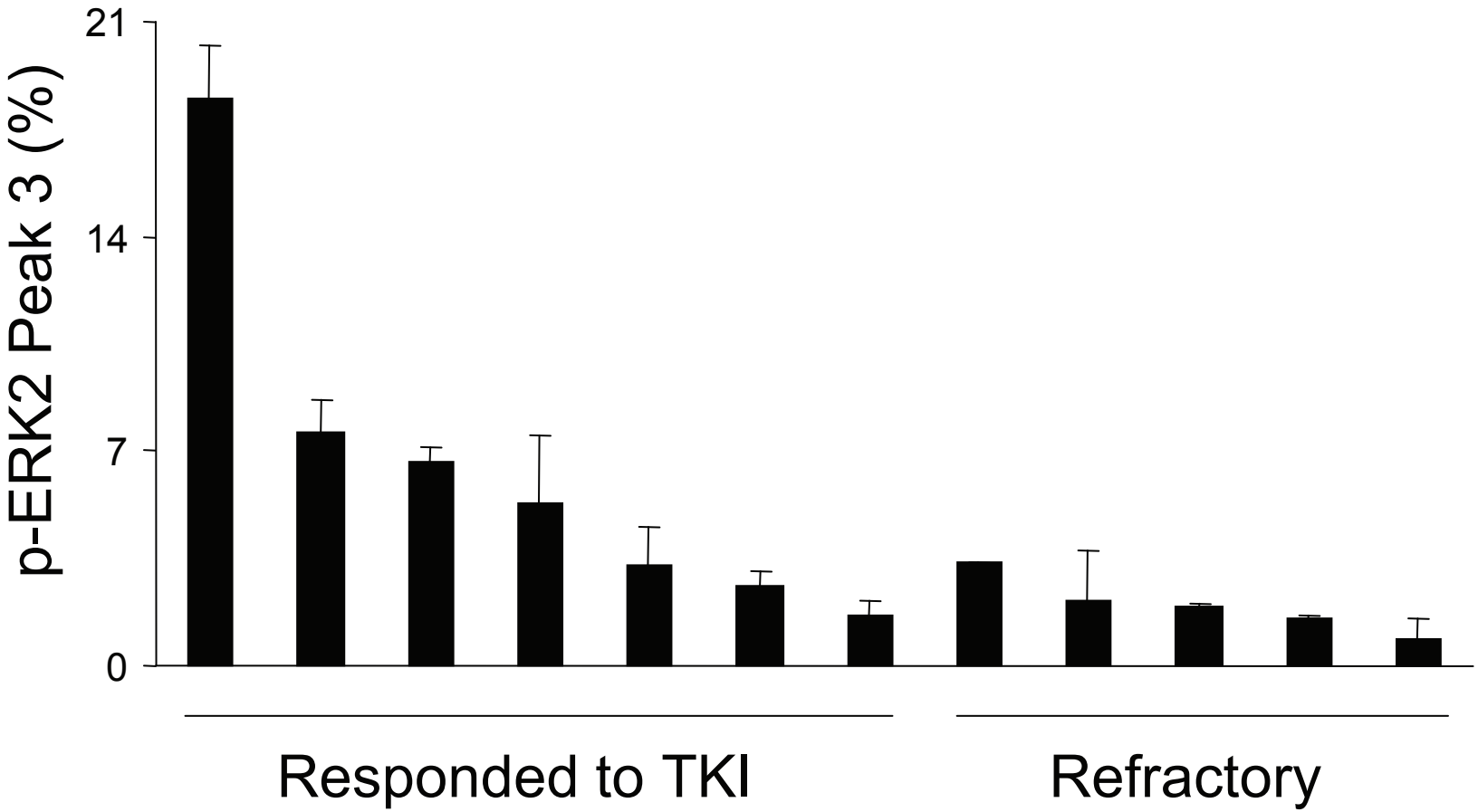
Supplementary figure 3. NIA Peak Identification of MEK1/2 Isoforms. Antibodies specific for total MEK1 (black traces), total MEK2 (green traces), pMEK1 (blue traces) and pMEK2 (red traces) were used to probe K562 cell line using NIA.



Supplementary figure 4. NIA Analysis of STAT3 Phosphorylation Following Imatinib Treatment in vitro. pSTAT3 was measured by NIA or FACS in K562 cells treated with imatinib or PBS for 0, 4, 8, 12, 16, 20 or 24h in vitro.



Supplementary figure 5. NIA Identifies a Decrease in a Specific Phospho-ERK Isoform upon Imatinib Treatment in vitro and in vivo. a. NIA detected a change in phospho-ERK in vitro in the K562 CML cell line after treatment with 24 hours with imatinib (peak 3, blue box). pERK was analyzed by Western blot and FACS b. Peripheral blood tumor cells were isolated from a CML patient before and after initiating imatinib treatment. NIA demonstrates the eradication of pERK2 peak 3 in vivo in a CML patient treated with imatinib. Representative NIA traces obtained in triplicate are shown. c. NIA Analysis of Total versus Phospho-ERK in CML Patients Treated with Tyrosine Kinase Inhibitors in vivo. Phospho-ERK was measured by NIA using an antibody that detects total (black trace) versus phospho-specific ERK antibody (blue trace). Phospho-ERK2 peak 3 is highlighted by the box.



Supplementary figure 6. Comparison of Basal pERK2 in Patients with CML .

Basal p-ERK2 values are graphed for 7 patients that subsequently responded to TKI ("responded to TKI"), and 5 patients that subsequently did not respond to TKI treatment ("refractory"). There is no statistically significant difference between the mean values for each group (unpaired t-test, 2 tailed, p= 0.1678). Mean value for each patient +/- SEM of experiments performed in triplicate are graphed.

Supplementary Table 1

			Best Response			
Patient	**Untreated WBC**	**Treated WBC**	**Hematologic***	**Cytogenetic***	**Molecular***	**pERK isoform**
1	585.0	5.7	Complete	Minor	N/A	Decrease
2	80.4	6.1	Complete	Complete	Major	Decrease
3	430.0	5.0	Complete	N/A	N/A	Decrease
4	9.3	2.3	Complete	N/A	N/A	Decrease
5	21.8	5.9	Complete	Partial	Major	Decrease
6	62.4	4.7	Complete	N/A	Complete	Decrease
7	106.0	5.0	Partial	N/A	N/A	Decrease
8	40.1	55.3	Refractory	Refractory	Refractory	No change
9	6.0	35.6	Relapse	Relapse	Relapse	Increase

* Response is defined based on guidelines from *Faderl S et al. Ann Intern Med 1999*
N/A= not available

Supplementary Methods

Recombinant proteins. MYC protein (Active Motif, Carlsbad, CA) or BCL2 protein (R&D Systems, Minneapolis, MN) were added to lysis buffer.

Tissue culture. Tumor derived cell lines were grown in RPMI (GIBCO) media containing 10% FCS, 1% penicillin/streptomycin (GIBCO), and 0.000003% betamercaptoethanol (Sigma). Cells were grown in T25 flasks in incubators maintained at 37 degrees with 5% carbon dioxide.

Imatinib treatment in vitro. 100 mg Imatinib (Novartis, Basel, Switzerland) tablets were dissolved in sterile PBS at 37°C and added to media for a final concentration of 10mM *in vitro*.

Conditional oncogene expression. All experiments were performed with the approval from the Stanford University Administrative Panel on Laboratory Animal Care. The TRE-MYC transgenic line generated for these experiments was described previously [1]. The TRE-BCL2 transgenic lines generated for these experiments were generated in conjunction with R. Padua. The Eμ-tTA transgenic line was kindly provided by H. Bujard [2]. Mice were mated and screened by PCR. Tumor derived cell lines were generated. Syngeneic mice were injected subcutaneously with lymphoma-derived cell lines containing tet-regulatable MYC or BCL2. When tumors reached > 1 cm^3, oncogene expression was suppressed *in vivo* by injecting mice with 100 μg of doxycycline in PBS IP

and adding doxycycline (100 μg/ml) to the drinking water. *In vitro*, MYC or BCL2 oncogenes were inactivated in tumor derived cell lines by the addition of doxycycline (0.01, 0.05, 2, or 20 ng/ml, final concentration) to the media.

Tumor sampling of transgenic tumors by Fine Needle Aspiration (FNA). We performed serial fine needle aspiration procedures (FNAs) on mice to obtain cell samples from subcutaneous lymphoma tumors before and three days after oncogene inactivation. Continuous negative pressure was applied to a 2 ml syringe with 20 gauge needle while 10 passes were made through the subcutaneous tumor. Specimens were collected into PBS. Red blood cells were removed using Pharmalyse (BD). Each FNA procedure obtained an average of 7 million cells.

Western. Western analysis was performed using conventional techniques [3]. Lymphoid tissues were disrupted and protein was isolated in HNTG lysis buffer [20mM Hepes pH 7.5, 25mM NaCl, 0.1%, 0.1% Triton X-100, 10% glycerol, Sigma Phosphatase Inhibitor Cocktail 1, Calbiochem Protease Inhibitor], then sonicated for 30 seconds, 5 minutes on ice (repeated twice) [4]. CML cells and CML patient specimens were lysed in RIPA lysis buffer RIPA lysis buffer [25mM Hepes pH 7.5, 150mM NaCl, 1% NP 40, 0.25% Na-deoxycholate, 10% glycerol, Sigma Phosphatase Inhibitor Cocktail 1, Calbiochem Protease Inhibitor]. 50ug protein was loaded in each lane, as quantitated by the Bicinchoninic Acid Protein Assay (Pierce). Proteins were electrophoresed on 10% Tris-HCl polyacrylamide

gels at 100 V for 60 min and transferred on PVDF membranes at 100 V for 60 min. The membrane was blocked in 5% nonfat dry milk solution in TBS at 4-8°C overnight. Blots were incubated with primary antibodies at 4-8°C overnight. MYC, BCL2, ERK, p-ERK, activated caspase 3, p-MEK1, pMEK1, pSTAT5, pJNK and HSP70 were detected with the same primary antibodies used for NIA. Blots were washed three times with TBST and then incubated for one hour with secondary anti-mouse or anti-rabbit HRP-conjugated antibodies (Amersham). ECL detection kit (Amersham) was used for antibody detection. The western data was quantitated using Image Quant and Data Acquisition & Analysis Version 7.3 software (Van Mierlo).

Phospho-protein FACs. Cells were fixed in 1.6% paraformaldehyde at 37°C for 10 minutes, permeabilized with 100% methanol for 10 minutes at room temperature, then washed with PBS, centrifuged at 2000 RPM for 5 minutes, subsequently washed with PBS 1% BSA, then resuspended in 50 ul of PBS 1% BSA. 1 million cells per FACs tube were stained with 10 ul anti-Phospho-ERK1/2 (Thr202/Tyr204):PE (BD Biosciences Pharmingen) in 100 ul PBS 1% BSA. After a 30 minute incubation in the dark, samples were washed once with PBS 1% BSA and subsequently analyzed using a benchtop FACSCAN (Becton-Dickinson) flow cytometer. 10,000 ungated events were collected per sample.

Supplementary Methods References

1. Felsher, D.W. & Bishop, J.M. Reversible tumorigenesis by MYC in hematopoietic lineages. *Mol Cell* **4**, 199-207 (1999).
2. Kistner, A.*, et al.* Doxycycline-mediated quantitative and tissue-specific control of gene expression in transgenic mice. *Proc Natl Acad Sci U S A* **93**, 10933-10938 (1996).
3. Towbin, H., Staehelin, T. & Gordon, J. Electrophoretic Transfer of Proteins from Polyacrylamide Gels to Nitrocellulose Sheets: Procedure and Some Applications. *PNAS* **76**, 4350-4354 (1979).
4. O'Neill, R.A.*, et al.* Isoelectric focusing technology quantifies protein signaling in 25 cells. *Proc Natl Acad Sci U S A* **103**, 16153-16158 (2006).